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Relocation of the t-SNARE SNAP-23 from Lamellipodia-like Cell Surface Projections Regulates Compound Exocytosis in Mast Cells

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Summary

For regulated secretion, mast cells and several other cell types utilize compound exocytosis, a combination of granule–plasma membrane and granule–granule fusions. The molecular machinery that controls this massive export process has not been identified. We report that SNAP-23, a t-SNARE related to SNAP-25, relocates in response to stimulation from plasma membrane lamellipodia-like projections to granule membranes in permeabilized mast cells. While relocation is a prerequisite for secretion, it can occur without membrane fusion and will expedite a subsequent secretory response. After relocation, SNAP-23 is required for exocytosis, implying a crucial role in promoting membrane fusion. Thus, relocation of this SNARE regulates compound exocytosis and links granule–plasma membrane and granule–granule fusions.

Introduction

Mast cells are highly specialized secretory cells that contribute to host defense by discharging a variety of proinflammatory mediators, chemotactic factors, and immunoregulatory cytokines (Abraham and Malaviya, 1997). Upon activation, mast cells typically release their large populations of secretory granules, often within a few minutes (Hide et al., 1993). Granule discharge takes place by compound exocytosis, a process that also occurs in other cells of hematopoietic origin and in certain endocrine and exocrine cells (Palade, 1959; Alvarez de Toledo and Fernandez, 1990). It is distinguished from unitary granule (or vesicle) exocytosis as typifies neurotransmitter release in that it involves a combination of granule–granule and granule–plasma membrane fusions and encompasses most if not all of the storage granule pool. Although the molecular events involved in compound exocytosis are unknown, ultrastructural and electrophysiological studies have suggested that the major physiological process involves sequential fusions of individual granules (Anderson et al., 1973; Alvarez de Toledo and Fernandez, 1990). It occurs as a cascade beginning at the cell surface, and fusion between granules is contingent on one of the fusion partners already having fused with the plasma membrane. Thus, the plasma membrane appears to play a key role in conferring "fusability" to storage granule membranes.

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Information regarding the molecular machinery of exocytosis in mast cells is limited. Some of the small GTP-binding proteins and associates, Rab3 (Oberhauser et al., 1992), Rac and Rho (Norman et al., 1996), Cdc42 (Brown et al., 1998), Rac/Rho-GDI (Mariot et al., 1996; O'Sullivan et al., 1996), and at least one heterotrimeric G protein, G_{i3} (Aridor et al., 1993), have been implicated as regulators; however, little is known about their specific roles, particularly with respect to compound exocytosis. There is even less information available regarding how the SNARE complex, which plays an essential role in mediating membrane trafficking (including neurotransmitter release) (Rothman, 1994; Sudhof, 1995; Hay and Scheller, 1997), contributes to membrane fusion events in mast cells. The key proteins comprising the prototype SNARE complex in neurons (Sollner et al., 1993) are synaptobrevin/VAMP 2 (v-SNAREs), syntaxin 1, and SNAP-25 (t-SNAREs). Each type of SNARE is now known to be conserved phylogenetically and to have one or more paralogs with broad distributions among mammalian cells and tissues (Ferro-Novick and Jahn, 1994; Bock and Scheller, 1997).

SNAP-23 was first identified in a human B lymphocyte cDNA library in a yeast two-hybrid screen for proteins that interact with syntaxin 4 (Ravichandran et al., 1996). Subsequently, others independently reported the identification of SNAP-23 in several cell types (Araki et al., 1997; Mollinedo and Lazo, 1997; Wang et al., 1997; Wong et al., 1997). The primary structure of SNAP-23 is 59% identical and 72% similar to that of SNAP-25; it contains a central cluster of cysteine residues that is a site of palmitoylation in SNAP-25 (Hess et al., 1992; Veit et al., 1996) and predicted coiled-coil segments that are thought to serve in binding other SNAREs, especially syntaxins 1, 3, and 4 (Ravichandran et al., 1996; Araki et al., 1997; Galli et al., 1998). SNAP-23, like SNAP-25, has been localized mainly to the plasma membrane (Araki et al., 1997; Gaisano et al., 1997; Wang et al., 1997; Wong et al., 1997). Unlike SNAP-25, human SNAP-23 is not cleaved by botulinum neurotoxins A and E (Chen et al., 1997; Sadoul et al., 1997). In agreement with its putative function, SNAP-23 has been reported to replace SNAP-25 in the process of insulin secretion when it is overexpressed (Sadoul et al., 1997).

We have used streptolysin O (SL-O) permeabilized mast cells to address the mechanism of compound exocytosis. While we identify VAMP 2 and syntaxins 3 and 4 as candidate secretory v-/t-SNAREs, our focus is on SNAP-23, which is a key regulator of compound exocytosis by a novel process involving stimulated relocation. SNAP-23's movement serves as an essential link between membrane fusion at the cell surface and within the stored granule population.

Results

Focal Concentration of SNAP-23 along the Plasma Membrane of Unstimulated Cells

For exploring the presence and role of SNAP-23 in mast cells, we used an antibody against its carboxy-terminal peptide. The antibody detects an ~29 kDa band in mast cell lysates that comigrates with the lone band present

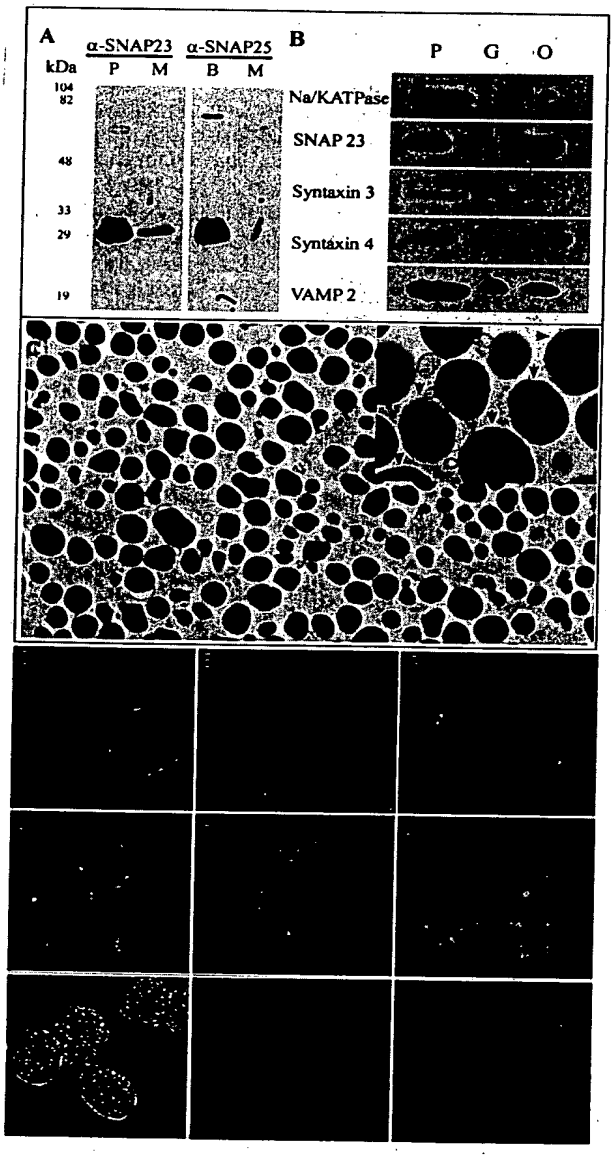


Figure 1. Mast Cells Contain SNAP-23, which Is Not Detected in Secretory Granules but Is Focally Concentrated at the Plasma Membrane of Unstimulated Cells

(A) Western blots of mast cells and control tissues for SNAP-23 and SNAP-25. Extracts of 2×10^5 mast cells (M) and crude membrane preparations (~10 μg protein) from parotid (P) and brain (B) were blotted with the indicated antibodies.

(B) Western blots of mast cell fractions (purified granules [G] and other membranes [O]) and crude membranes from parotid (P). Loads are equal portions of each fraction.

(C) Low-power EM of purified granules. The fraction contains intact granules with limiting membranes (arrows) and some small vesicles (inset).

(D–L) Localization of SNAREs in mast cells by confocal immunofluorescence. For SNAP-23 staining, cells were prepared by 3 different methods: (1) fixation of intact cells before staining (D); (2) permeabilization with SL-O followed by fixation and staining (E); and (3) permeabilization with SL-O and no fixation before staining (F). For syntaxin 3 (G), syntaxin 4 (H), and VAMP 2 (I) staining, procedure 3 was used. Phase contrast image (J) and controls for immunostaining (K and L) were performed using procedure 3. (K) Samefield as in (J); incubation with SNAP-23 epitope peptide was performed before antibody staining. (L) Immunostaining with primary antibody omitted.

in parotid membranes (Figure 1A), 3T3-L1, CHO, and Vero cells (data not shown). The antibody does not cross-react with SNAP-25 in rat brain membranes (where it is enriched; data not shown). Conversely, SNAP-25 is not detected in mast cells using a SNAP-25–specific antibody (Figure 1A, right).

To begin to address the function of SNAP-23 in mast cell secretion, we investigated its subcellular distribution, as well as that of other SNARE proteins. We used cell fractionation to separate intact granules as a purified fraction from other membranous organelles (including broken granules). The granule fraction contained 70% of the total β-hexosaminidase activity (a secretory enzyme) of the homogenate, confirming that the fraction is representative, and examination by EM indicated that it is highly enriched for membrane-bound granules (Figure 1C and inset). Minor contamination includes membraneless granule cores and occasional small vesicles. Western blotting for Na/K ATPase (α subunit) showed the presence of a single band in the "other membrane" fraction, with none in the granule fraction (Figure 1B), indicating that contamination of the granules by plasma membrane was not detected. Blotting with antibodies against SNAP-23 and other SNAREs showed that SNAP-23 is highly concentrated in the other membrane fraction and is not detected in granules (Figure 1B). Syntaxin 4 exhibited the same distribution. In contrast, VAMP 2 and syntaxin 3 were clearly detected in both fractions.

We localized SNAP-23 in mast cells using confocal immunofluorescence microscopy. SNAP-23 was highly concentrated at the cell periphery with little detected in the cell interior (Figures 1D–1F). The observed staining is specific, as it was blocked by excess epitope peptide (Figures 1J and 1K), and no staining was observed when primary antibody was omitted (Figure 1L). Localization along the plasma membrane is consistent with previous observations for SNAPs 23 and 25 (Oyler et al., 1989; Wang et al, 1997). Notably, SNAP-23 in mast cells was largely concentrated in focal spots along the cell border. These foci were also observed in intact cells that were fixed initially (Figure 1D) and in permeabilized and fixed cells (Figure 1E). Therefore, they are unlikely to be related to use of SL-O- or to antibody-mediated aggregation/cross-linking.

Staining of permeabilized mast cells with antibodies to other SNAREs is also shown in Figures 1G–1I. Like SNAP-23, syntaxin 4 is concentrated in foci along the cell border (Figure 1H). In contrast, the staining of syntaxin 3 (Figure 1G) and VAMP 2 (Figure 1I) is distributed throughout the cytoplasm, consistent with the presence of both of these polypeptides in secretory granule membranes (Figure 1B).

Concentration of SNAP-23 in Lamellipodia-like Projections and Association with the Triton-Insoluble Cytoskeleton

We were curious whether the SNAP-23 foci corresponded to cell surface specializations or subplasmalemmal vesicles. Consequently, we used immunoelec-

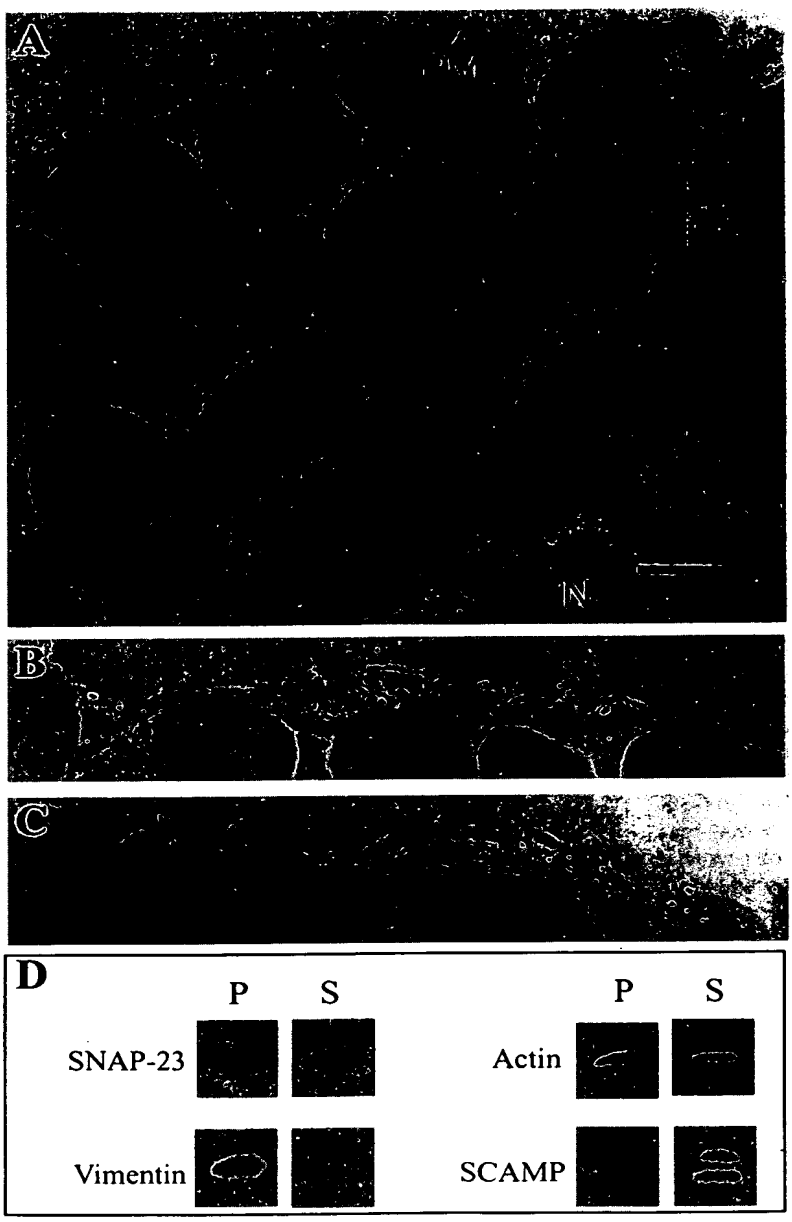


Figure 2. EM Immunogold Localization of SNAP-23 in Unstimulated Mast Cells and Association of SNAP-23 with the Triton-Insoluble Cytoskeleton

(A–C) Examples showing that SNAP-23 is highly concentrated in surface lamellipodia-like projections with little intracellular labeling. PM, plasma membrane; N, nucleus. Bar = 0.5 μm.

(D) Western blot illustrating that a substantial amount of SNAP-23 sediments with the Triton-insoluble cytoskeleton. S, supernatant (half of total); P, pellet (total).

tron microscopy on permeabilized cells to examine this issue. Representative images (Figures 2A–2C) show a striking clustering of SNAP-23 on the cytoplasmic aspects of membrane protrusions, small folds, and spikes that resemble lamellipodia and cover the surface of intact mast cells (Hide et al., 1993). Quantitation of labeling, conducted in a more extensive study presented below, indicated that two-thirds of plasmalemmal projections were labeled, and that they accounted for 77% of cell surface–associated gold particles.

As cell surface projections like lamellipodia are actin rich (Nobes and Hall, 1995), we wondered whether SNAP-23 might be associated with the cortical cytoskeleton that persists in SL-O-permeabilized mast cells (Koffer et al., 1990; Norman et al., 1996). To begin to explore this possibility, we used a procedure applied in other hematopoietic cells (Fox et al., 1988) and examined whether SNAP-23 was resistant to solubilization by Triton X-100. Results shown in Figure 2D indicate that a substantial amount of the total SNAP-23 was not solubilized, but was cosedimented with most of the actin and all vimentin. In contrast, an integral membrane protein (SCAMP; Brand and Castle, 1993) was fully solubilized and did not sediment. Following Triton treatment, we also adjusted the suspension to 40% sucrose and loaded it beneath lower concentration sucrose layers for density gradient centrifugation (Sargiacomo et al., 1993). Western blots of gradient fractions indicated that none of the SNAP-23 floated upward, but instead remained in the load or sedimented (data not shown). This

analysis ruled out significant association of SNAP-23 with detergent-insoluble glycolipid complexes, a conclusion that is also supported by lack of codistribution of SNAP-23 and the GPI-anchored surface antigen Thy1, as determined by immunofluorescence (data not shown).

GTPγS or Calcium Elicits Relocation of SNAP-23 that Must Precede or Parallel Compound Exocytosis

To examine the function of SNAP-23 in compound exocytosis in mast cells, we modified established procedures (Gomperts and Tatham, 1992; Koffer and Churcher, 1993) for stimulating SL-O-permeabilized cells, using single effectors of secretion, either GTPγS or Ca^{2+}. Notably, we increased the dose of SL-O (1.6 IU/ml instead of 0.4 IU/ml) to enable internalization of anti-SNAP-23 antibody as a functional perturbant. In our version of the permeabilized system (Figure 3A), we were consistently able to trigger release of ~50% and ~30% of total hexosaminidase activity by buffered Ca^{2+} (10 μM) and GTPγS (100 μM), respectively, in the presence of ATP.

As SNAP-25 has been implicated to function at a late step in fusion (Banerjee et al., 1996; Mehta et al., 1996; Rossi et al., 1997), we reasoned that SNAP-23 acts similarly and wondered whether its action might include both granule–plasma membrane and granule–granule fusions. If so, the progression of compound exocytosis should be linked to relocation of SNAP-23 to the fusion sites. Thus, we examined SNAP-23 immunostaining in stimulated cells. As shown in Figures 3B–3E, we observed a striking redistribution of SNAP-23 that was closely coupled to exocytosis. Redistribution involved two processes (Figures 3C and 3E): (1) dispersion from the foci at the cell surface to give more extended segments of intense plasma membrane staining, and (2) relocation to intracellular sites throughout the cytoplasm. The latter staining mostly appeared in the form of uniform rings corresponding in size to granule profiles and suggested that SNAP-23 might have relocated to granule membranes. Relocation required Ca^{2+} and ATP (Figure 3C) or GTPγS and ATP in the presence of cytosol (Figure 3E); ATP alone (Figure 3B), Ca^{2+} alone (data not shown), or cytosol plus ATP (Figure 3D) did not elicit it. Thus, conditions that stimulate exocytosis were needed for relocation.

The correlation between relocation of SNAP-23 and secretion does not establish a functional link between the events, especially as it is unclear whether relocation is a prerequisite to secretion, happens to occur in parallel, or is contingent on membrane fusion. In addressing these possibilities, we first sought to establish whether the two processes could be uncoupled, as relocation without secretion would rule out a dependence on membrane fusion. As shown in Figures 4B–4G, when permeabilized mast cells were incubated with Ca^{2+} and ATP for 30 min on ice and then returned to Ca^{2+}-free medium by centrifugation and resuspension, translocation of SNAP-23 was clearly revealed by immunostaining. However, no exocytosis had occurred, as judged by enzymatic assay of secreted hexosaminidase (Figure 4A) and by electron microscopy (e.g., Figure 4H). The pattern of

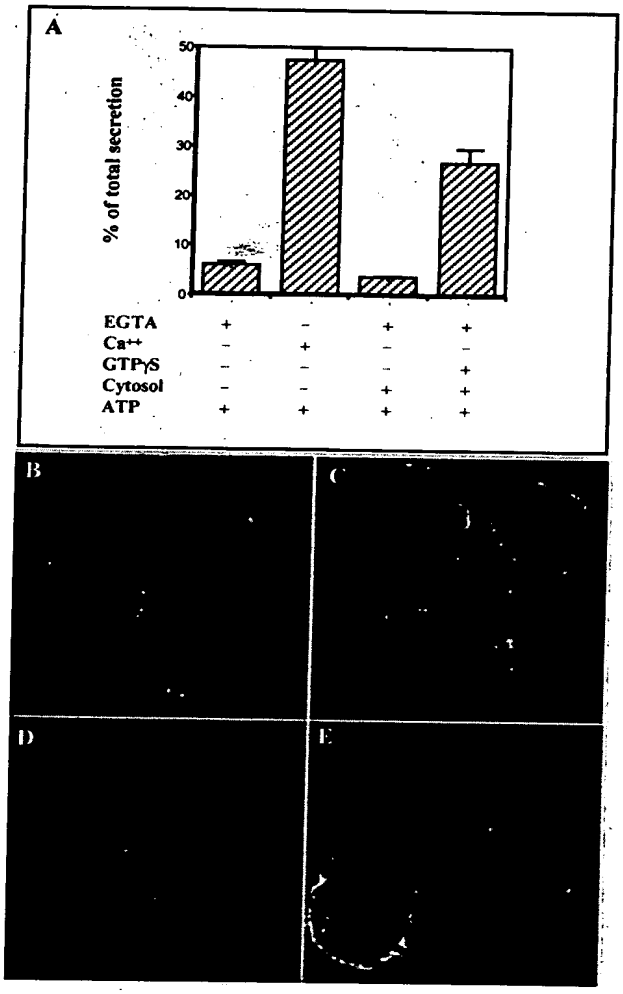
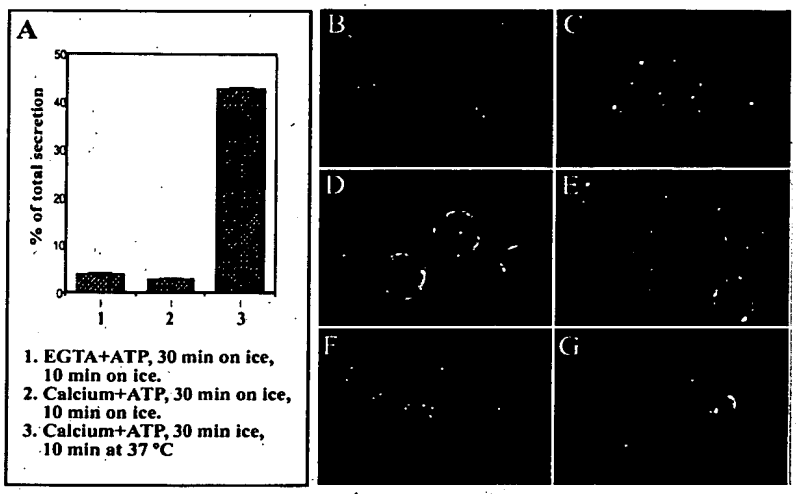


Figure 3. Relocation of SNAP-23 in Mast Cells in Response to GTPγS or Calcium Stimulation Is Correlated with Mast Cell Secretion

Permeabilized cells were treated under various conditions, as indicated in (A) or specifically as follows: (B) EGTA + ATP, (C) Ca^{2+} + ATP, (D) EGTA + ATP + cytosol, (E) EGTA + ATP + cytosol + GTPγS. (A) Hexosaminidase secretion; (B–E) immunofluorescence of SNAP-23.

immunostaining observed after low temperature relocation (Figures 4D and 4G) was similar to what was observed with 37°C incubation (Figure 3C), and relocation at low temperature exhibited the same requirement for the combined presence of Ca^{2+} and ATP (Figures 4B and 4D). To ensure that SNAP-23 relocation took place during the low temperature incubation and not during subsequent manipulations, we capitalized on the ability of the anti-SNAP-23 antibody to block relocation (see below). Accordingly, we compared the distributions of SNAP-23 when antibody was added before and after the 30 min incubation at low temperature in the presence of Ca^{2+}/ATP. Where antibody was added in advance, no translocation was observed (Figure 4F); however, where added after 30 min, translocation had clearly taken place (Figure 4G).



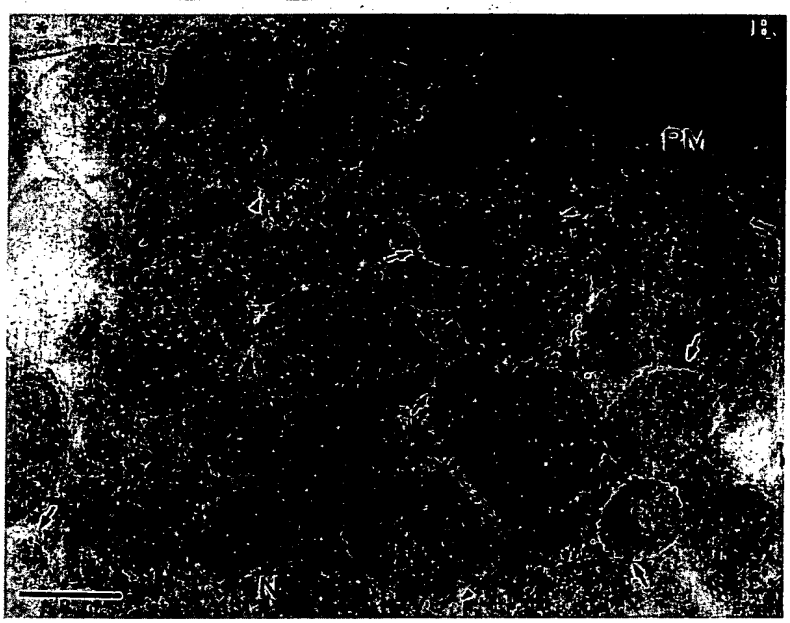


Figure 4. Uncoupling Relocation of SNAP-23 from Secretion Using Ca^{2+}/ATP at Low Temperature

(A) Permeabilized cells were incubated as indicated and assayed for secretion.

(B–G) Permeabilized cells were incubated 30 min on ice with EGTA + ATP(B), Ca^{2+} (C), Ca^{2+} and ATP (D, E, and G), or EGTA and SNAP-23 antibody (F). Following washing, cells in (F) were incubated with Ca^{2+} + ATP and cells in (G) with EGTA and SNAP-23 antibody for another 30 min on ice. Subsequently, cells in (B)–(D), (F), and (G) were stained with anti-SNAP-23 antibody, whereas cells in (E) were stained with syntaxin 4 antibody.

(H) EM immunogold localization of SNAP-23 to granule membranes and other intracellular sites after treatment with Ca^{2+}/ATP at low temperature. Cells were incubated as in (D), and immunogold labeling was performed as in Figure 2. Labeling appears on granule membranes (arrows) and other structures (arrowheads mark examples and correspond to "other sites" in Table 1). Labeling remains on the plasma membrane (PM, *). Bar overlying the nucleus (N) = 0.5 μm.

Two controls were included to discount the possibilities that the intracellular staining observed after Ca^{2+}/ATP treatment reflected nonspecific antibody binding or unmasking of cryptic epitopes. First, immunostaining was blocked entirely by excess epitope-containing peptide (data not shown) just as for unstimulated cells (Figure 1K), thereby ruling out nonspecific binding. Second, cells with and without Ca^{2+}/ATP treatment at low temperature were immunostained with anti-SNAP-23 and fluorescent secondary antibody and then examined by both immunofluorescence microscopy and fluorescence-activated cell sorting (FACS). By microscopy, we determined that relocation had occurred in 70% of the cells, and the FACS analysis showed that the fluorescence intensity profiles were identical (data not shown). Therefore, we concluded that bona fide relocation, rather than unmasking of cryptic SNAP-23, was being detected.

SNAP-23 is thought to interact with the t-SNARE syntaxin 4 (Ravichandran et al., 1996; Araki et al., 1997), which spans the bilayer (Bennett et al., 1993). We were interested to examine whether syntaxin 4 accompanied SNAP-23 during relocation, especially into the cell interior, as low temperature is thought to preclude a mechanism involving membrane fusion. When anti-SNAP-23 antibody was replaced with anti-syntaxin 4 antibody after washing the cells at the end of Ca^{2+}/ATP treatment at low temperature, we observed no intracellular staining (Figure 4E). Instead, syntaxin 4 retained a focal pattern of concentration at the cell surface. Apparently, relocation of SNAP-23 at low temperature does not involve membrane fusion or vesicular trafficking.

We used immuno-EM to examine the distribution of SNAP-23 after relocation had been induced at low temperature. An example is shown in Figure 4H, and Table

Table 1. EM Immunogold Distribution of SNAP-23 Label in Permeabilized Mast Cells

	Unstimulated (EGTA)	Stimulated (Ca^{2+} ATP)
Plasma membrane	72.2 (±2.1)	31.1 (±1.8)
Intracellular structure		
Granule membrane	5.2 (±0.7)	28.0 (±1.6)
Granule content	1.0 (±0.2)	0.7 (±0.2)
Nuclear envelope	2.8 (±0.4)	2.0 (±0.4)
Nuclear matrix	2.6 (±0.5)	6.3 (±1.0)
Other sites	16.2 (±1.8)	31.9 (±1.7)

SL-O-permeabilized mast cells were incubated with EGTA or Ca^{2+}ATP for 30 min on ice and then labeled with anti-SNAP-23. 2412 gold particles from 31 randomly sampled unstimulated mast cells and 2530 gold particles from 32 randomly sampled stimulated mast cells were counted. Gold particles on granule content, nuclear envelope, and matrix probably reflect background labeling. "Other sites" correspond to unidentified intracellular membranes and amorphous structures.

1 presents quantitative results for antigen distribution before and after relocation. As seen from the micrograph, intracellular gold is readily detected in many cases closely apposed to the surfaces of secretory granules. Gold is also detected on less well-defined structures ("other sites" in Table 1) that are interspersed among the granules with only occasional labeling on the nuclear envelope and matrix and granule content. Some labeling remains at the cell surface as expected from the immunofluorescence results. In all immuno-EM experiments, we included a control in which incubation with primary antibody was omitted (as for immunofluorescence, Figure 1L). Under these circumstances, only rare gold particles were detected.

Quantitation of immunogold labeling (Table 1) showed that intracellular labeling accounted for about one-quarter of the total in unstimulated cells and almost 70% after the low temperature Ca^{2+}/ATP treatment. In unstimulated cells, about half of the intracellular label was attributed to unidentified structures, including a portion occasionally seen as a perinuclear concentration, while about one-fifth of the intracellular label was found on granule membranes. Following relocation, labeling on granule membranes increases more than 5-fold, while labeling of other (unidentified) sites doubles. Significantly, the extent of redistribution to the cell interior is large enough so that it is possible to deduce that the antigen must have redistributed from the surface projections to the internal organelles.

In efforts to gain insight as to whether SNAP-23 dissociates and is solubilized during relocation, and also whether it stably binds to granule membranes after relocation, we tested for leakage from permeabilized cells at increasing times of exposure to Ca^{2+}/ATP at either 0°C or 37°C, and we fractionated the treated cells to test for both nonsedimentable and granule-associated antigen by Western blotting. Leakage of SNAP-23 from permeabilized cells was not detected, and no antigen was detected in soluble fractions during organelle purification. Further, granule-associated SNAP-23 also was not detected in five attempts, even though we routinely

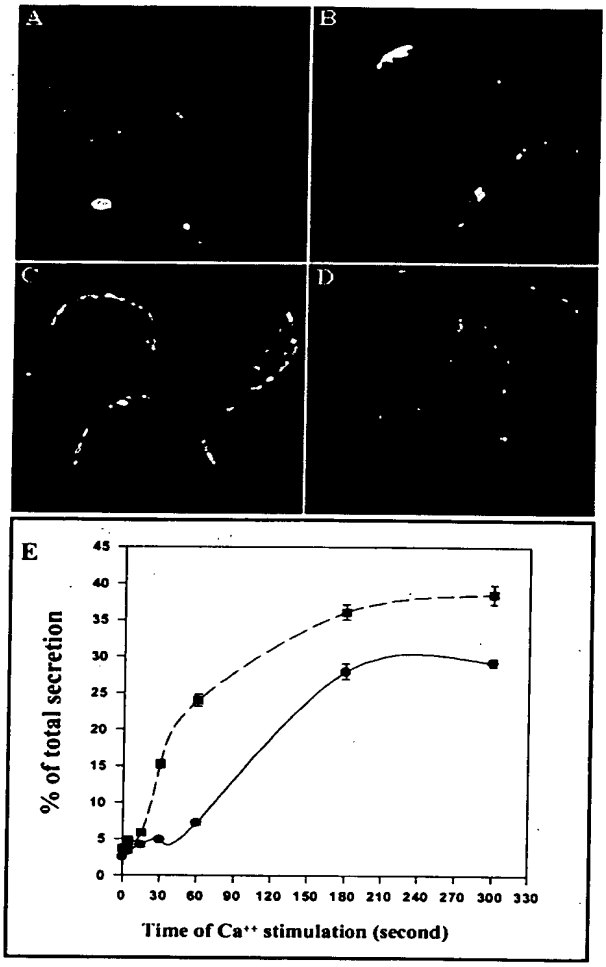


Figure 5. Relocation of SNAP-23 Precedes and Facilitates Compound Exocytosis

(A–D) Time course of Ca^{2+}-stimulated relocation of SNAP-23 at 37°C: 5 s (A), 15 s (B), 60 s (C), and 600 s (D).

(E) Time course of hexosaminidase secretion stimulated by Ca^{2+}/ATP without or with advance relocation of SNAP-23 at low temperature. Two sets of permeabilized cells were incubated 30 min on ice with either GB (circles) or with added Ca^{2+} and ATP to induce relocation (squares). Subsequently, Ca^{2+}/ATP was added at the same final concentrations to the buffer-alone set of samples, and both sets were immediately incubated at 37°C for the indicated times.

recovered half of the total granules from the permeabilized cells, as measured by hexosaminidase activity (data not shown). So far, we are unable to distinguish among the possibilities that relocation to the granule surface is incomplete under our conditions, that relocation occurs but is unstable and doesn't survive fractionation, or that granules with bound SNAP-23 are unstable and lyse.

Relocation of SNAP-23 Precedes and Expedites Compound Exocytosis

Low temperature uncoupling has convincingly demonstrated that relocation of SNAP-23 is not dependent on secretion; however, it has not distinguished whether

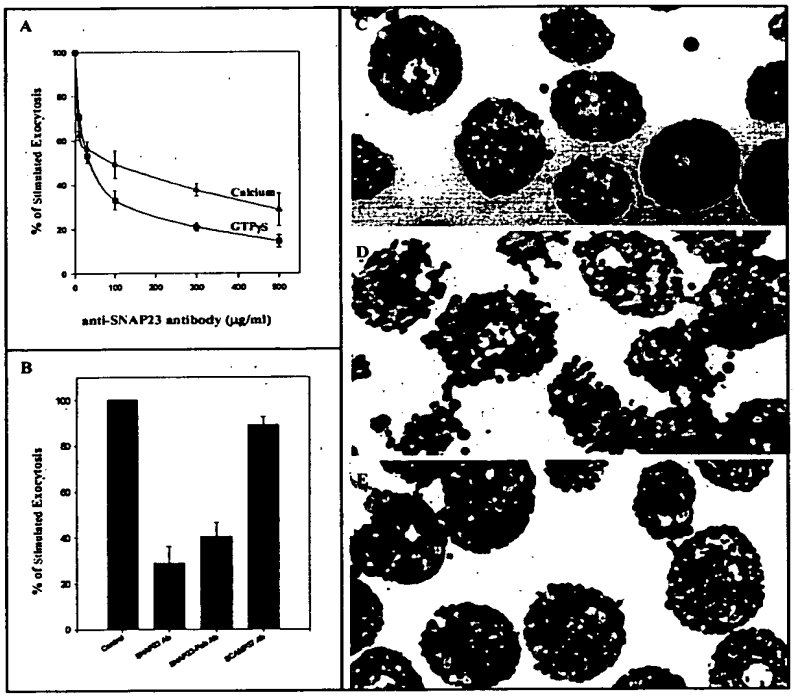


Figure 6. SNAP-23 Is Required for Compound Exocytosis in Mast Cells

(A) Dose curves for inhibition of GTPγS- or Ca^{2+}-triggered secretion by SNAP-23 antibody. Cells were treated as in Figure 5E except that antibody was included during low temperature incubation. For GTPγS stimulation, cells were washed to remove cytosol before warming to test secretion.

(B) SNAP-23 antibody and its Fab fragment inhibit Ca^{2+}-stimulated secretion. Permeabilized cells were incubated with Ca^{2+}, ATP, and the indicated antibody (500 μg/ml) for 30 min on ice and 10 min at 37°C. Hexosaminidase release is normalized to that of stimulated samples lacking antibody.

(C–E) Light micrographs (0.5 μm sections, stained with 0.5% toluidine blue) showing inhibition of compound exocytosis by SNAP-23 antibody. (C), EGTA + ATP + cytosol; (D), EGTA + ATP + Cytosol + GTPγS; and (E), EGTA + ATP + cytosol + GTPγS + SNAP-23 antibody. Decreased intracellular staining and extracellular granule cores (D) indicate compound exocytosis.

relocation precedes or coincides with secretion. This distinction is important, as relocation in advance of secretion would suggest a possible role as a prerequisite for compound exocytosis. We carried out two types of experiments to address the relative timing of relocation and secretion. First, we compared their time courses from 5 s to 10 min at 37°C. As shown in Figures 5A–5D, the relocation of SNAP-23 elicited by Ca^{2+}/ATP was both rapid and progressive through the cell population. At 5 s (Figure 5A), it was well advanced in a few cells, but not in most, whereas unstimulated cells showed no relocation. By 15 s (Figure 5B), the population of cells exhibiting relocation had increased significantly, and relocation had spread more thoroughly throughout the cell interior. By 1 min (Figure 5C), nearly all cells exhibited relocation, and the distribution of SNAP-23 did not appear to change further at 10 min (Figure 5D). On the other hand, assays of hexosaminidase (Figure 5E, circle/solid line) at 5 s, 15 s, and 1 min indicated that secretion of only 3%, 4%, and 7%, respectively, of the total had been detected. Further, as secretion triggered by calcium is an all-or-none event in individual cells (Hide et al., 1993), the number of cells showing degranulation is much less than the number showing relocation of SNAP-23. Therefore, we concluded that relocation occurred more rapidly.

In the second type of experiment, we capitalized on our uncoupling procedure and compared the kinetics of hexosaminidase secretion on samples stimulated with Ca^{2+}/ATP at 37°C with and without prior relocation of SNAP-23 at low temperature. As shown in Figure 5E (circle/solid line), without prior relocation there is a delay before the onset of rapid and massive exocytosis. This is characteristically observed when mast cells are induced to undergo exocytosis using single effectors (GTPγS or Ca^{2+}) in either permeabilized or patch-clamped cells (Koffer, 1993; Oberhauser et al., 1996). In contrast, when SNAP-23 relocation was induced initially at low temperature, the delay was greatly reduced (Figure 5E, square/broken line). Evidently, this result shows that advance relocation of SNAP-23 expedites secretion, suggesting that relocation precedes secretion. Importantly, we acknowledge that relocation of SNAP-23 is not the only event that might take place at low temperature, and the increased kinetics of secretion that we have observed may not necessarily result from relocation of SNAP-23. However, the observation that SNAP-23 is required for secretion, especially after relocation (see below), directly links relocation of SNAP-23 to secretion and strongly argues that relocation of SNAP-23 is a prerequisite for exocytosis, particularly compound exocytosis.

Relocation Is Required for Secretion, and SNAP-23 also Functions in Exocytosis after Relocation

To explore further the apparent dependence of compound exocytosis on SNAP-23 relocation, we introduced our anti-SNAP-23 antibody into SL-O-permeabilized cells and examined its effect on subsequent stimulated secretion of hexosaminidase. As shown in Figure 6A, the antibody substantially inhibited both Ca^{2+}- and GTPγS-triggered secretion in a concentration-dependent manner. Inhibition was specific on several accounts. First, complete removal of unbound antibody by centrifugation and resuspension in fresh stimulatory medium did not alter inhibition (data not shown). Second, substitution of anti-SNAP-23 by a rabbit antibody against SCAMP1 (Wu and Castle, 1997), which detects antigen both on

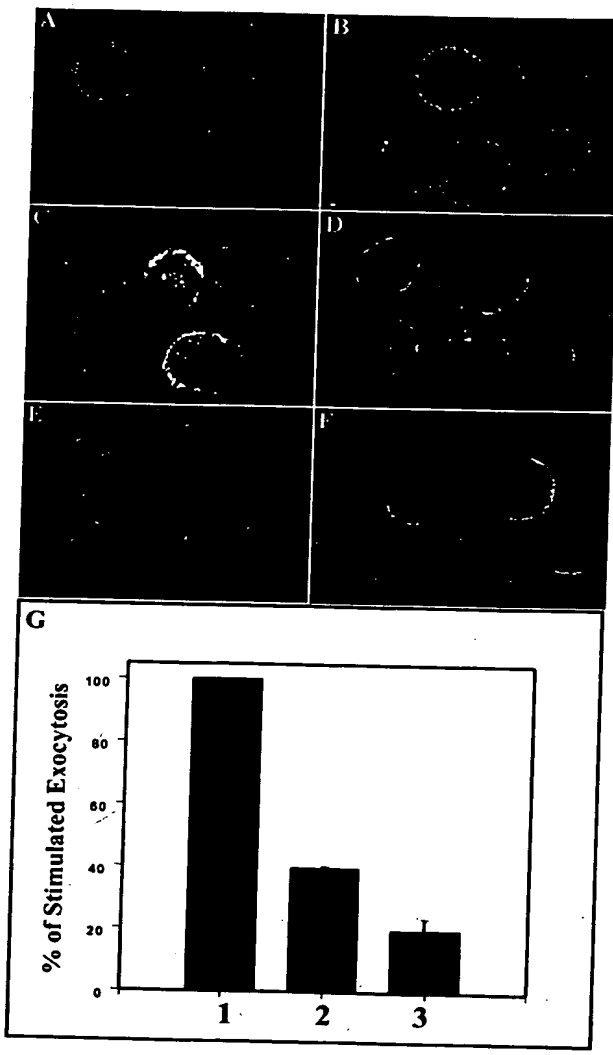


Figure 7. Relocation of SNAP-23 Is Inhibited by SNAP-23 Antibody, and SNAP-23 Is Also Required for Secretion after Relocation

(A–F) Permeabilized cells were incubated 30 min on ice with the following ingredients: (A), EGTA + ATP + cytosol; (B), EGTA + ATP; (C), EGTA + ATP + cytosol + GTPγS; (D), Ca^{2+} + ATP; (E), EGTA + ATP + cytosol + SNAP-23 antibody (500 μg/ml); and (F), Ca^{2+} + ATP + SNAP-23 antibody (500 μg/ml). All samples were then incubated at 37°C for 10 min before SNAP-23 immunostaining.

(G) SNAP-23 antibody inhibits secretion before and after relocation of SNAP-23. Permeabilized cells were incubated 30 min on ice as follows: (1) Ca^{2+} + ATP; (2) ATP + SNAP-23 antibody (500 μg/ml); and (3) Ca^{2+} + ATP. Subsequently, Ca^{2+} was added to (2), and SNAP-23 antibody was added to (3) (same final concentrations), and incubation of all samples was continued for 30 min on ice prior to triggering secretion at 37°C for 10 min.

granules and at cell surface sites where SNAP-23 is concentrated (data not shown), has no inhibitory effect on secretion (Figure 6B). Third, substitution of anti-SNAP-23 by its Fab fragment still blocks secretion (Figure 6B). Finally, we confirmed that the inhibition measured by enzymatic assay of released hexosaminidase reflects inhibition of exocytosis as judged by light microscopy (Figures 6C–6E) and EM (data not shown). The morphological results, in particular, illustrate that the antibody interferes with granule–plasma membrane as well as granule–granule fusions.

In considering the mechanisms by which anti-SNAP-23 might inhibit exocytosis, we examined whether the antibody affected relocation of SNAP-23 by adding it to permeabilized cells before stimulating secretion and then examining the outcome by immunofluorescence. As shown in Figures 7A–7F, antibody clearly prevented translocation, correlating closely with inhibition of secretion (Figure 6). We also noticed that the effect of the antibody differed, depending on whether relocation was triggered by GTPγS or by Ca^{2+}. In the case of GTPγS, the antibody inhibited both dispersion of SNAP-23 along the cell surface and penetration intracellularly (Figures 7A, 7C, and 7E), whereas in the case of Ca^{2+} stimulation, the antibody only inhibited intracellular penetration, not surface movement (Figures 7B, 7D, and 7F). The differing effect on relocation in response to the two stimuli agrees quite well with the inhibition of secretion in that the antibody is more potent in blocking exocytosis stimulated by GTPγS than by Ca^{2+} (Figure 6A).

The latter study showed that the antibody perturbed exocytosis, especially compound exocytosis, by preventing relocation of SNAP-23. However, to test whether the antibody might also block secretion following relocation, we induced relocation of SNAP-23 using Ca^{2+}/ATP at low temperature, then added anti-SNAP-23 antibody while maintaining the same levels of Ca^{2+} and ATP, and finally triggered exocytosis by warming to 37°C. Inhibition was still observed and possibly was stronger than when anti-SNAP-23 was added prior to relocation (Figure 7G). Taken together, these findings imply that relocation of SNAP-23 is not only a prerequisite for secretion, but also that once relocated, SNAP-23 plays an essential role in the final stages of membrane fusion.

Discussion

While mast cells have been one of the signature cell types displaying massive and rapid compound exocytosis in response to secretory stimulation, the identity of the machinery that controls these events, especially the propagation of membrane fusion through the granule population, has remained obscure. Our studies provide evidence for a critical role of SNAP-23 and suggest that it confers "fusability" to at least certain types of secretory granules as a prerequisite to discharge.

The focal concentration of SNAP-23 along the mast cell surface (Figure 1) resembles what has been reported for SNAPs 23 and 25 elsewhere (Oyler et al., 1989; Jacobson et al., 1994; Kannan et al., 1996; Wang et al., 1997), although foci are less evident in some cell lines (Araki et al., 1997; Sadoul et al., 1997; Wong et al., 1997). The concentration sites correspond to plasmalemmal projections or folds (Figure 2) that resemble stable lamellipodia. This localization may be related to the association of SNAP-23 with apical microvilli in intestine-derived cells (Galli et al., 1998). Based on our findings that relocation of SNAP-23 is a prerequisite for mast cell secretion (Figures 4–6), we propose a novel function for these surface specializations in sequestering exocytotic machinery. Because SNAP-23 is concentrated in

actin-rich surface projections and is substantially resistant to Triton extraction (Figure 2), we presume that the cytoskeleton may function in sequestration and that mobilization following stimulation may be linked to cortical cytoskeletal rearrangement. Indeed, Ca^{2+}/ATP treatment dissociates the cortical cytoskeleton in permeabilized mast cells (Koffer et al., 1990). Moreover, recent studies have shown that both secretion and rearrangement of the cortical cytoskeleton in mast cells is regulated by members of the Rho family of GTPases—Cdc42, Rac, and Rho (Price et al., 1995; Norman et al., 1996; Brown et al., 1998). It is well known that Cdc42, Rac, and Rho control the organization of filopodia, lamellipodia, and focal adhesions (Nobes and Hall, 1995). Therefore, it is reasonable to speculate that these G proteins, as well as Ca^{2+}/ATP, are acting to mobilize SNAP-23 and/or to drive the relocation process.

How might a membrane-anchored protein like SNAP-23 relocate? Three possibilities come to mind: the first is diffusion along the membrane interface; the second is dissociation from the plasma membrane by deacylation or by binding to a cytosolic chaperone and reattaching at distant sites; and the third is translocation along the reorganizing cytoskeleton. These possibilities are not mutually exclusive. Since SNAP-23 is putatively palmitoylated, it may relocate by diffusion along the membrane interface. This pathway may explain redistribution of SNAP-23 in the plasma membrane but is unlikely to account for movement into the cell. Although hemifusion (Kemble et al., 1994) between the cytoplasmic leaflets of the granule and plasma membranes offers a potential pathway for diffusion into the cell along membrane interfaces, this route is unlikely for two reasons. First, hemifusion would have to occur on a massive scale at low temperature; and second, a surface diffusion pathway does not explain the increased presence of specific staining on internal nongranule structures (Table 1). We also don't favor a pathway involving dissociation of SNAP-23 from the plasma membrane into the cytosol and reattachment to intracellular membranes because leakage from permeabilized mast cells with or without stimulation is negligible, and SNAP-23 has not been detected in cytosolic fractions derived from control cells or from cells that have been stimulated 0–30 min at either 4°C or 37°C. Our preliminary evidence showing that a large fraction of SNAP-23 is Triton-insoluble and cosediments with actin and vimentin (Figure 2) supports the last possibility that relocation of SNAP-23 may be linked with rearrangement of the cytoskeleton. This possibility may also be supported by our observation that relocation of SNAP-23 requires ATP and Ca^{2+} (Figure 3), which may implicate a motor-driven process. Further investigation of these events is needed and is a high priority.

The movement of SNAP-23 into the cell interior (Figures 3–5 and 7), where a substantial portion appears associated with granule membranes (Figure 4H; Table 1), suggests that SNAP-23 may play a role in fusion events within the granule population in addition to its presumed role at the plasma membrane. This suggestion is supported by our finding that advance relocation greatly reduces the lag between stimulation and release (Figure 5E) and by the thorough inhibition of secretion by anti-SNAP-23 antibody when added after relocation (Figure 7). What might be the possible roles of SNAP-23? In view of its abilities to substitute for SNAP-25 (Sadoul et al., 1997) and to form SNARE complexes (with syntaxin 3 and a VAMP 2 relative [Galli et al., 1998]), relocated SNAP-23 may enable formation of ternary SNARE complexes within individual granules that then facilitate SNARE activation by NSF and α-SNAP (Otto et al., 1997; Ungermann and Wickner, 1998). Further, as several studies suggest that SNAP-25 and homologs function in fusion beyond the SNARE activation step (Banerjee et al., 1996; Mehta et al., 1996; Rossi et al., 1997; Ungermann and Wickner, 1998), SNAP-23 may do likewise. This later role may involve formation of intermembrane complexes between granules and their targets (plasma membrane or other granules); however, the precise role remains to be identified.

The recognized role of SNAP-23/25 family members in facilitating activation of SNAREs for fusion provides additional insight regarding the significance of SNAP-23 relocation. As secretory granules in unstimulated mast cells lack SNAP-23 (Figure 1), formation of ternary SNARE complexes is not possible until SNAP-23 relocates. This implies that relocation of SNAP-23 controls entry of mast cell granules into the "fusion-ready" state. Indeed, the absence of fusion-ready granules in mast cells has been noted previously (Oberhauser et al., 1996), as expressed by the lag that characteristically occurs between stimulation and detectable secretion. Our data (especially Figure 5E) argue that relocation of SNAP-23 is strongly correlated with moving granules into a fusion-ready pool.

The role of sequestration and relocation of SNAP-23 in regulating the fusion-ready pool of secretory carriers may be broadly applicable to other cell types, including those that use SNAP-25 to control the release of hormones and neurotransmitters. In contrast to mast cells, endocrine cells and neurons maintain steady-state populations of dense core granules and synaptic vesicles that are fusion-ready and undergo immediate release with no lag following stimulation (Parsons et al., 1995; Heidelberger, 1998). Notably, portions of both endocrine granule and synaptic vesicle populations maintain SNAP-25 on their surfaces (Walch-Solimena et al., 1995; Hohne-Zell and Gratzl, 1996; Tagaya et al., 1996). Based on our insight from SNAP-23, we suggest that this localization is a prerequisite to achieving the fusion-ready state involving SNARE activation and subsequent regulatory interactions that precede fusion. Consequently, sequestering SNAPs 23 and 25 at the cell surface and restricting levels on secretory vesicles may be a general mechanism for controlling entry into the fusion-ready vesicle pool. Thus, while some of the SNAP-23/25 on the surface may function as a bona fide plasma membrane t-SNARE, another portion may serve as a reserve pool for relocation.

Overall, our findings have provided the beginnings of a model for the mechanism and regulation of compound exocytosis at the molecular level. This model employs analogs of the SNAREs that have been almost universally invoked to function in expediting membrane fusion in intracellular trafficking. However, the mechanism is orchestrated by regulated relocation of one of the SNAREs, SNAP-23.

Experimental Procedures

Antibodies

Rabbit anti-SNAP-23 antibody was raised (Covance Inc., Denver, PA) against a C-terminal peptide, (C)ANARAKKLIDS, and was affinity purified on peptide-Sulfolink resin (Pierce Chemical Co.). Other antibodies were kind gifts: rabbit anti-syntaxins 3,4 (Drs. Pam Tuma and Ann Hubbard, Johns Hopkins Medical School); rabbit anti-SNAP-25 (Dr. Pietro DeCamilli, Yale School of Medicine); monoclonal anti-VAMP 2, C1 69.1 (Dr. Reinhard Jahn, Max Planck Institute, Gottingen, Germany); monoclonal anti-Na/K ATPase α-subunit 6H (Dr. Michael Caplan, Yale School of Medicine). Rabbit anti-SCAMP1 (1Ω) and monoclonal anti-SCAMP were previously described (Wu and Castle, 1997). Monoclonals, anti-actin, and anti-vimentin were from Amersham Inc. (Arlington Heights, IL) and Santa Cruz Inc. (Santa Cruz, CA), respectively.

Mast Cell Purification and Subcellular Fractionation

Rat mast cells were purified from peritoneal and pleural cavities by an established method (Gomperts and Tatham, 1992). Following incubation (5 min, 24°C) with 5 mM DFP (diisopropylfluorophosphate, Sigma), mast cells were homogenized by sonication, and subcellular fractions were purified on Percoll gradients (Kruger et al., 1980; Chock and Schmauder-Chock, 1989).

Reconstitution of Regulated Exocytosis in SL-O-Permeabilized Cells

Mast cells were permeabilized and stimulated using modifications of established methods (Gomperts and Tatham, 1992; Koffer, 1993). Cells (2×10^5) in 1.5 ml ice-cold GB buffer (137 mM K *l*-glutamate, 2 mM $MgCl_2$, 20 mM Pipes, 1 mg/ml BSA [pH 6.8]) were washed by centrifugation (250 × g, 4°C, 1–2 min) and resuspended to 500 μl in GB containing 3 mM EGTA and 1.6 IU/ml SL-O (Murex Co.). Following gentle mixing, cells were incubated 1.5 min, 37°C for permeabilization, immediately chilled, and washed twice with 1.5 ml GB at 4°C. Following resuspension in 200 μl GB, 10 μl (1×10^4 cells) aliquots were added to 40 μl GB plus different combinations of the following reagents (according to the particular experiment; see Results and figure legends): 3 mM EGTA; 10 μM Ca^{2+} (CaEGTA buffer; Gomperts and Tatham, 1992); 1 mM ATP (GTPγS stimulation) or 3 mM ATP (Ca^{2+} stimulation); 500 μg/ml purified rat brain cytosol (Miller and Moore, 1992); and the indicated concentrations of antibody in GB. Cells were kept 30 min on ice for peptide or antibody uptake and then incubated at 37°C (0–10 min, as specified) to stimulate secretion. Reactions were terminated by chilling and centrifugation. Supernatants (10 μl) and cells lysed in Triton X-100 (total) were assayed for hexosaminidase (Gomperts and Tatham, 1992). Results were expressed as percent total secretion using means and standard errors from at least three different experiments. Error bars were calculated using an unpaired t test (StatView program).

Immunofluorescence Microscopy

Following permeabilization and stimulation as described above, 50 μl of 32% goat serum in GBE (GB, 3 mM EGTA) was added to block nonspecific binding. After 30 min, 100 μl of primary antibody diluted with 16% serum in GBE was added to achieve 100 μg/ml anti-SNAP-23, 8 μg/ml anti-syntaxin 4, 10 μg/ml anti-syntaxin 3, 1:100 dilution of anti-VAMP 2 ascites fluid, and 100 μg/ml anti-SCAMP1 in the respective samples. Following 30 min incubation, cells were washed three times with GBE and were incubated with 200 μl 16% goat serum in GBE containing Cy3-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, 1:400 dilution). Finally, cells were washed four times with GBE and visualized by confocal microscopy (Zeiss LSM 410; Thornwood, NY). When fixed before staining, intact or SL-O-permeabilized cells were treated with 3% formaldehyde in 0.15 M phosphate (pH 7.4) for 30 min at 24°C, incubated further with 0.2 μM digitonin (Sigma) for 5 min, and then blocked with goat serum (5% in GBE) and stained with anti-SNAP-23 (5–10 μg/ml). Images of cells shown are representative of over 90% of the total.

Immunoelectron Microscopy

EM immunostaining was performed on permeabilized and unfixed cells as described above except that secondary antibody was replaced by a goat anti-rabbit Fab fragment conjugated to 1.4 nm colloidal gold (Nanoprobes Inc., Stony Brook, NY) and diluted 1:100 in GBE containing 16% goat serum. Following secondary antibody staining, cells were washed, fixed overnight on ice in 3% glutaraldehyde in 0.2 M phosphate (pH 7.5), and then 30 min more in 1% glutaraldehyde, 0.2% tannic acid in 0.2 M phosphate. Samples were washed four times in 200 mM sucrose, 50 mM HEPES (pH 5.8) and then subjected to silver enhancement (HQ Silver™ Enhancement Kit, Nanoprobes Inc.) for 6 min (V = 100 μl) according to the manufacturer's instructions. Enhancement was stopped by adding 0.5 ml 250 mM sodium thiosulfate, 20 mM HEPES (pH 7.4) (Burry et al., 1992), and samples were washed three times in 200 mM sucrose, 50 mM HEPES (pH 5.8) and transferred to "acid histamine buffer" (Fernandez et al., 1991) to preserve granule condensation. After 30 min on ice, samples were dehydrated sequentially in 50%, 70%, 90%, and 100% ethanol (each except 100% diluted to volume with acid histamine buffer), equilibrated into SPURR resin, and embedded. Thin sections were stained 2.5 min with 1:10 dilution of Millonig's lead acetate and examined by EM.

For quantitating immunogold labeling, individual sections were collected at 10 μm intervals and mounted one section per grid. Random whole cells that included a nuclear profile were photographed (one per grid hole) in full cross-section for each experimental condition. Gold counts were performed manually, and the percent of total label for different organelles was calculated for each cell. Standard errors were calculated as for histamine assays. In a separate analysis, the distribution of gold at the cell surface was analyzed by counting gold particles and lamellipodia-like surface projections.

Extraction of Mast Cells with Triton X-100 and Analysis

DFP-treated, permeabilized mast cells (2×10^5) were resuspended in 100 μl extraction buffer (0.5% Triton X-100, 100 mM KCl, 20 mM HEPES [pH 7.3], 10 mM EGTA, and proteinase inhibitors [AEBSF, PMSF, leupeptin]), maintained 30 min at 0°C, and centrifuged for 10 min at 10,000 × g. Pellets were washed once in the same buffer by recentrifugation. The resulting supernatants and pellets were analyzed by Western blotting (Wu and Castle, 1997).

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